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Antimicrobial Susceptibility Testing of *Francisella tularensis* with a Modified Mueller-Hinton Broth

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A modified Mueller-Hinton broth was developed to perform antimicrobial susceptibility tests on *Francisella tularensis*. Adequate growth of the organism was obtained within 24 h of inoculation, and MICs could be read at that time. We tested 15 selected strains of *F. tularensis* and five reference quality control strains in this medium with 36 antimicrobial agents. The MICs of the aminoglycosides and tetracycline increased 1 to 3 dilutions in this medium compared with those in the usual medium, but the other antimicrobial agents were not consistently affected by the medium. Even though the medium caused an increase in MICs, the aminoglycosides and tetracyclines remained very active in vitro against *F. tularensis*. Other antimicrobial agents effective in vitro were chloramphenicol, erythromycin, ceftazidime, moxalactam, cefotaxime, ceftriaxone, and Sch 29482 (a cephalosporin).

Francisella tularensis, a bacterium primarily transmitted to humans by wild mammals and biting arthropods, causes human infections which may be prolonged and difficult to treat. The bacteria can be entrapped by the reticuloendothelial system, where the organisms are capable of surviving intracellularly for long periods of time (3, 11, 22). Streptomycin, tetracycline, and chloramphenicol have, for many years, been the recommended antimicrobial agents of choice for treating this infection (3, 12, 21). There is disagreement as to which antimicrobial agent is most efficacious for the disease since there are frequent relapses when the course of treatment is completed. Some authors (4, 18, 22, 25) have reported high relapse rates when tetracycline was used. The organisms from these patients had not become resistant to tetracycline but merely survived intracellularly, and relapse occurred after cessation of therapy (22). Other investigators reported tetracyclines to be as effective as streptomycin when the appropriate course of therapy was given (14, 18, 22). Penicillin, ampicillin, and cephalothin have been used to treat this infection, usually before the infecting agent was identified, and generally were not effective (4, 11, 12, 24).

In addition to streptomycin, tetracycline, and chloramphenicol, other antimicrobial agents reported to be active against *F. tularensis* are kanamycin, gentamicin, and novobiocin (4, 12, 18). In vitro susceptibility testing of *F. tularensis* has not been routinely or systematically performed because the bacterium is difficult to grow on conventional laboratory medium and because of the danger of laboratory workers acquiring laboratory infections. Some nutrients required for growth of the organisms tend to inactivate some antimicrobial agents (1, 5, 7, 23). In addition, the organism produces ammonia, which changes the pH of the medium and may result in changes in the in vitro susceptibility to some antimicrobial agents and may cause the growth in the medium to become self-limiting (23). This study was done to: (i) develop a medium that would support the growth of *F. tularensis* and would be acceptable for antimicrobial susceptibility testing, (ii) determine the susceptibility of *F. tularensis* to a variety of antimicrobial agents, both old and new, which might be considered for treatment of *F. tularensis* infections, and (iii) add as much information about organism-antimicrobial agent combinations as possible while these highly infectious organisms were being tested.

MATERIALS AND METHODS

The 36 antimicrobial agents that were tested are listed in Tables 1 and 2. Reference antimicrobial powders were supplied by the respective manufacturers. These anitmicrobial agents were prepared for testing as directed in the National Committee for Clinical Laboratory Standards M7-T standard (15).

The medium that best supported growth of *F. tularensis* and was used for susceptibility tests consisted of Mueller-Hinton broth supplemented with calcium and magnesium ions (15), 0.1% glucose, 2% IsoVitaleX (BBL Microbiology Systems) which contains cysteine, and 0.025% ferric pyrophosphate. All supplements were added aseptically after the Mueller-Hinton broth was sterilized at 121°C for 15 min. The pH was adjusted to 6.9 to 7.1 with sterile 1 N NaOH after all supplements had been added.

Fifteen strains of *F. tularensis* were selected from the culture collection of the Special Bacteriology Laboratory, Centers for Disease Control. They consisted of 13 glycerol-positive and 2 glycerol-negative strains. All strains were β-lactamase positive by the nitrocefin test incubated for 1 h (24). Two strains were selected because they had been grown on modified charcoal-yeast extract agar before submission to the Centers for Disease Control and because these organisms are sometime mistaken for *Legionella* spp.(10). One strain, KC1482, derived from strain ATCC 6223 (5, 7, 8, 17) was selected for testing because it is the avirulent type strain and it grows poorly and slowly on conventional laboratory media. These strains had been isolated from wounds, blood, conjunctiva, pleural fluid, and lymph nodes. Most of these isolates were from the southeastern and southwestern areas of the United States.

The organisms were grown on GC chocolate agar (BBL) in a candle extinction jar for 24 to 48 h. Suspensions of approximately 10^8 CFU/ml (equivalent to a 0.5 McFarland

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TABLE 1. MICs of β-lactam antimicrobial agents against *F. tularensis*

Antibiotic	MIC (μg/ml)		
	Range	50%[a]	90%[b]
Penicillin	4.0->8.0	>8.0	>8.0
Ampicillin	>8.0	>8.0	>8.0
Oxacillin	≤0.06->2.0	>2.0	>2.0
Methicillin	≤0.12->4.0	>4.0	>4.0
Carbenicillin	≤2.0->256.0	>256.0	>256.0
Ticarcillin	>64.0	>64.0	>64.0
Mezlocillin	16.0->64.0	>64.0	>64.0
Piperacillin	≤0.5->64.0	64.0	64.0
Azlocillin	16.0->64.0	>64.0	>64.0
Cephalothin	≤0.25-8.0	>8.0	>8.0
Cefamandole	8.0->16.0	>16.0	>16.0
Cefoxitin	≤0.25-16.0	4.0	8.0
Cefotetan	0.5->32.0	8.0	>32.0
Cefotaxime	≤0.12-4.0	2.0	4.0
Moxalactam	≤0.12-2.0	≤0.12	0.25
Cefoperazone	1.0->32.0	16.0	>32.0
Ceftazidime	≤0.5-1.0	≤0.5	≤0.5
Ceftriaxone	0.5-16.0	2.0	8.0
Aztreonam	4.0->32.0	16.0	>32.0
Sch 29482	≤0.25->32.0	2.0	4.0

[a] MIC at which 50% of the strains were inhibited.
[b] MIC at which 90% of the strains were inhibited.

standard) of these organisms were prepared in the modified Mueller-Hinton broth and further diluted 1:50 in the same medium. Microdilution trays containing the antimicrobial agents diluted in modified Mueller-Hinton broth were inoculated from this final suspension of the test organism with disposable inoculators (0.01 ml; Dynatech Laboratories, Inc.). The number of viable organisms was determined for the growth control well of the inoculated microdilution tray by colony counts. The final inoculum averaged 2×10^6 CFU/ml (2×10^5 CFU per well) and ranged from 9.6×10^5 to 3.5×10^6 CFU/ml. The inoculated microdilution trays were incubated in candle extinction jars for 24 h at 35°C. The MIC was read as the lowest concentration of antimicrobial agent which inhibited visible growth of the organisms.

All manipulations of the organisms were done in a laminar flow biologic safety cabinet, and protective clothing, gloves, and masks were worn.

Parallel microdilution trays were prepared with cation-supplemented Mueller-Hinton broth (15). Control strains *Staphylococcus aureus* ATCC 25923 and ATCC 29213, *Streptococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were tested in both media. The control cultures were tested in the same manner as *F. tularensis* in the modified Mueller-Hinton broth and tested as described by the National Committee for Clinical Laboratory Standards M7-T in the cation-supplemented Mueller-Hinton broth (15).

RESULTS

Several modifications of Mueller-Hinton broth were tested for growth of *F. tularensis*. The supplements tested included lysed horse blood, IsoVitaleX, glucose, ferric pyrophosphate, hemin, and calcium and magnesium ions. The combination which gave adequate growth of *F. tularensis* within 24 h was Mueller-Hinton broth supplemented with Ca^{2+} and Mg^{2+} (15), 2% IsoVitaleX, 0.1% glucose, and 0.025% ferric pyrophosphate. Blood, cysteine, peptone, and glucose have been reported to be required for growth of the organism (1, 5, 7, 9, 13, 16, 19, 20, 23). We used ferric pyrophosphate because two of these organisms were isolated or grew on charcoal-yeast extract agar (6). Growth on the same medium supplemented with lysed horse blood or hemin instead of the ferric pyrophosphate was very poor and was insufficient for antimicrobial susceptibility testing. When the iron source was omitted from the medium, *F. tularensis* did not grow. We did not test hemoglobin because a clear medium was needed to facilitate reading the MICs.

The MICs for which 50% of the organisms were inhibited (MIC_{50}s) and MIC_{90}s are shown in Table 1. In general, the penicillins were not very active on these strains of *F. tularensis* but note that the ranges of MICs for the penicillins are quite wide and that MIC_{50}s are high. The main reason for the wide range is that one strain had low MICs for some of these β-lactam antimicrobial agents. This unusual strain had been sent to the Centers for Disease Control as a suspect *Legionella* sp. because it grew on charcoal-yeast extract agar. Even though it had low MICs to some penicillins, this strain was β-lactamase positive.

The cephalosporins were more active, as a group, than the penicillins against these strains. Cephalothin and cefamandole were the least active, and moxalactam, ceftazidime, cefotaxime, ceftriaxone, and cefoxitin were the most active cephalosporins.

All of these strains of *F. tularensis* were β-lactamase positive by the nitrocefin method in 1 h (24).

Table 2 shows the MICs for the aminoglycosides, all of which were active in vitro. Other active antimicrobial agents were tetracycline, erythromycin, chloramphenicol, nitrofurantoin, and rifampin.

The in vitro effect of modified Mueller-Hinton broth on control strains compared with results obtained in Mueller-Hinton broth supplemented with Ca^{2+} and Mg^{2+} was minimal with the penicillins and cephalosporins. The greatest change was a decrease of 2 $\log_2$ dilution for ceftriaxone against *Streptococcus faecalis* ATCC 29212. All other results for the β-lactams were the same or within ±1 $\log_2$ dilution. This was not the case, however, for the other antimicrobial agents tested. The effect was greater for gentamicin, tobramycin, and netilmicin than for other drugs with MICs generally 2 to 3 $\log_2$ dilutions higher in modified Mueller-Hinton broth than in Mueller-Hinton broth sup-

TABLE 2. MICs of various antimicrobial agents against *F. tularensis*

Antibiotic	MIC (μg/ml)		
	Range	50%[a]	90%[b]
Streptomycin	≤0.5-4.0	2.0	4.0
Gentamicin	0.25-2.0	1.0	2.0
Tobramycin	≤0.12-4.0	1.0	2.0
Netilmicin	0.25-2.0	1.0	2.0
Amikacin	≤0.25-2.0	1.0	2.0
Fortimicin	0.25-4.0	2.0	4.0
Tetracycline	≤0.25-2.0	2.0	2.0
Chloramphenicol	≤0.25-4.0	1.0	1.0
Erythromycin	0.5-2.0	1.0	2.0
Clindamycin	1.0->2.0	>2.0	>2.0
Nitrofurantoin	≤2.0-16.0	≤2.0	8.0
Colistin	8.0->16.0	>16.0	>16.0
Rifampin	≤0.03-1.0	0.5	1.0
Vancomycin	>16.0	>16.0	>16.0

[a] MIC at which 50% of the strains were inhibited.
[b] MIC at which 90% of the strains were inhibited.

plemented with Ca^{2+} and Mg^{2+} (Table 3). This modified Mueller-Hinton broth was unsatisfactory for susceptibility testing of imipenem, sulfonamides, and trimethoprim.

An additional 15 strains of *F. tularensis* from an outbreak in South Dakota have been tested by this method, and the results were similar to the data reported here (manuscript in preparation).

DISCUSSION

This modification of Mueller-Hinton broth appears to be a satisfactory test medium for performing broth dilution susceptibility tests with *F. tularensis*. *F. tularensis* grows well in this medium in 24 h and can be easily seen in microdilution trays. The MICs are easily read and are reproducible. Caution must be exercised when interpreting antimicrobial results obtained in media that has been supplemented, e.g., the supplemented Mueller-Hinton broth used in these studies. The addition of metal ions results in higher MICs (i.e., antibiotics appear to be less active) for aminoglycosides and tetracyclines. Therefore, it is probable that these two groups of antimicrobial agents are more active in vitro against *F. tularensis* than our results would indicate, since the medium had additional metal ions. The addition of IsoVitaleX interferes with testing sulfonamides and trimethoprim, and indeed we found the medium unsuitable for testing these drugs against *F. tularensis*. The additional glucose would not create a problem in susceptibility testing unless excessive amounts of acid were formed, which would not seem to be the case since there was little effect on the erythromycin MICs.

Antimicrobial susceptibility tests with *F. tularensis* have been done by broth and agar dilution and by disk diffusion. Although disk diffusion tests have been reported (2, 18), we do not recommend the disk diffusion methods for two reasons. First, a disk diffusion method has not been recommended for these organisms, i.e., no correlations have been made between zones of inhibition and MICs, and no breakpoints have been shown to be accurate. Second, we believe the use of a disk method puts the user at greater risk of acquiring a laboratory infection with this organism because of a greater likelihood of spread of organisms through the air. Agar dilution tests have been used and reported (9, 12, 18; H. T. Eigelsbach, R. D. Herring, and T. W. Halstead, Bacteriol. Proc., p. 69, 1957). We would assume this method to be appropriate but, in general, it is less practical. If testing must be done, we prefer and recommend the microdilution method with the modified Mueller-Hinton medium described here. We do not recommend routine antimicrobial susceptibility testing for these organisms since empirical therapy seems appropriate and because of the safety hazard of working with these organisms. If susceptibility tests are required, they should be done by experienced personnel in laboratories with adequate safety devices, e.g., laminar flow hood, mechanical pipetters, and safety clothing.

Older antimicrobial agents, i.e., those that have been available for some time, in addition to streptomycin, tetracycline, and chloramphenicol, which appear to be active on *F. tularensis* by in vitro testing, include rifampin and erythromycin. In this study one strain was from a patient (suspected of having legionellosis) who had been treated with erythromycin and had responded to this therapy. This suggests that this antimicrobial agent might be useful in the treatment of tularemia. Newer antimicrobial agents which appear to be active against *F. tularensis* are the aminoglycosides gentamicin, tobramycin, netilmicin, amikacin, and fortimicin; the β-lactams cefotaxime, moxalactam, ceftazidime, and ceftriaxone; and Sch 29482, a cephalosporin. It is likely that these aminoglycosides could be used successfully to treat this infection since streptomycin has been shown to be efficaceous. The use of antimicrobial agents other than aminoglycosides, tetracycline, and chloramphenicol to treat patients with tularemia must await further data on clinical use.

In conclusion, if antimicrobial susceptibility tests on *F. tularensis* are done, we recommend using the microdilution method and the modified Mueller-Hinton broth. By this method, *F. tularensis* was susceptible in vitro to all the aminoglycosides tested, tetracycline, chloramphenicol, erythromycin, nitrofurantoin, and rifampin. With rare exceptions, penicillins were not active against these organisms, but the activity of the cephalosporins varied. Ceftazidime and moxalactam were the most active, but they were also susceptible to cefoxitin, cefotaxime, ceftriaxone, and Sch 29482.

TABLE 3. Log_2 dilution change in MICs of various antimicrobial agents for control strains tested in modified Mueller-Hinton broth compared with that in MICs tested in cation-supplemented Mueller-Hinton broth

Antibiotic	Control cultures (ATCC no.)[a]				
	S. faecalis (29212)	*S. aureus* (29213)	*S. aureus* (25923)	*E. coli* (25922)	*P. aeruginosa* (27853)
Streptomycin	—	+1	−1	+1	0
Gentamicin	≥+2[b]	+2	+1	+2	+2
Tobramycin	≥+2	+3	≥+3	+3	+2
Netilmicin	≥+3	+2	+1	+2	+1
Amikacin	—	+1	0	+1	+1
Fortimicin	—	+1	+1	+1	—
Tetracycline	—	+1	+1	+1	—
Chloramphenicol	0	0	0	0	—
Erythromycin	−1	0	0	—	—
Clindamycin	—	0	0	—	—
Nitrofurantoin	0	0	0	0	—
Colistin	—	—	—	−1	0
Rifampin	−1	—	—	+1	—
Vancomycin	0	0	−1	—	—

[a] Scale: 0, No change; −1, 1 dilution lower in modified Mueller-Hinton broth; +1, 1 dilution higher; +2, 2 dilutions higher; +3, 3 dilutions higher; —, results off scale, and change could not be determined.

[b] Results were off scale for one medium only.

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